5-1995

Membrane Protein as a Basis of NACL Tolerance in Alfalfa

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MEMBRANE PROTEIN AS A BASIS OF NACL TOLERANCE IN ALFALFA

by

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A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

in

Plant Science

UTAH STATE UNIVERSITY
Logan, Utah

1995

ABSTRACT

Membrane Protein as a Basis of NaCl Tolerance in Alfalfa

by

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Utah State University, 1995

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This study sought to determine whether NaCl altered the plasma membrane proteins in alfalfa exhibiting differential NaCl concentrations, and whether $CaSO_4$ modified the responses. Two alfalfa cultivars, Centurion and Condor, were grown in 0.5 strength Hoagland solution in a greenhouse. The cultivars were exposed to 0, 88, and 132 mM of NaCl alone and mixed with $CaSO_4.H_2O$ at 7 and 14 Mm $CaSO_4$ for 3, 9, and 60 days.

In experiment 1, roots were dried to determine their Na, Ca, K, and Mg concentration. The results were similar to previous reports in which $CaSO_4$ alleviated the salt stress by increasing K and Mg levels and reducing Na.

In experiment 2, after proteins of the plasma membrane were isolated and their purity was determined by vanadate, ATPase activity showed a significant increase in the presence of calcium. In addition, total plasma membrane protein was analyzed by sodium dodecylsulfate-polyacrylamide gel

electrophoresis. Salt treatments induced both quantitative and qualitative changes in proteins. These changes were affected by the length of exposure to treatment solution or the ability of the plants to adapt to the salt stress.

(88 pages)

To my parents

Who taught me patience, honesty, and virtue while working

ACKNOWLEDGMENTS

I would like to express my sincere thanks to my major professor, Dr. W. F. Campbell, for his guidance and support while directing this thesis.

I would like to thank my committee members, Dr. J. J. Jurinak and Dr. G. A. Long, for kind guidance and acceptance for serving on my committee. Also, I would like to express my thanks to Dr. D. V. Sisson for statistical analysis.

I would like to thank the Harriri Foundation for their financial support. I also wish to thank my father, Shieck Nizar Al-halabi, Mr. Riad Nashef, and Mr. Samir Tabish for their moral support and encouragement.

Thanks are also extended to my friends, Dr. T. S. Al-Niemi, G. O. Mikati, and S. M. Awada, for providing help during this work.

Husni Sabah

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INTRODUCTION

Salt-affected soils are an ever-increasing problem throughout the world's irrigated crop lands, affecting about 950 million hectares (28, 41). Lack of fresh water, rapid evaporation, poor drainage, and overuse of fertilizers on limited arable land are contributing factors to the continued accumulation of salts. The accumulated salts often contain sodium chloride (NaCl). The presence of excessive levels of Na^+ ions in the soil solution results in Na^+ ions on the exchange complex, which can cause deflocculation and deterioration of the physical structure of the soil, which reduces water infiltration, aeration, and root penetration (7). Also, the presence of excess Na-salts depresses plant growth by osmotic effects (water stress), which causes dehydration of plant cells or ion-specific effects (ion imbalance or toxicity) and inhibits the absorption of nutrients (59).

There have been two main ways to solve salinity/sodicity problems. One can either grow tolerant cultivars or one can add chemical amendments, e.g., $CaSO_4$ (62), that neutralize the harmful effects of Na^+ ions to plants (27, 55). Calcium is an important element in plants and plays a major role in membrane integrity and function, cell wall extension, and cellular stress recovery (42). Recent attention has focused on the Na^+-Ca^{2+} interactions in salt-stressed glycophytic crop plants and the role of Ca^{2+} in counteracting the effect of NaCl on plant

growth and other salt-related phenomena (15).

Ions enter cells by two major types of transport, passive and active. The passive transport of ions is a simple diffusion process. The active transport of ions is accompanied by ATP (adenosine triphosphate) hydrolysis against the gradient. ATPase is an enzyme system that plays a major role in regulating ion transport at the soil/root interface (85). The biochemical properties and proton pump activities of plasma membrane bound, ion-stimulated ATPase have been reported for a number of plant species under non-stressed conditions (58, 85). Recently, Suhayda et al. (84), working with NaCl-stressed and non-stressed tomato (Lycopersicon esculentum Mill.) roots, observed that NaCl-induced changes in electrostatic properties may influence ion transport across plasma membranes.

Little work has been done to determine the effect of salinity on plasma membrane protein and on the amelioration effect of $CaSO_4$ on membrane protein under NaCl stress. This study will focus on the relationship between Na^+ influx and the ion activity of Ca^{2+} in salinized solutions and with the emphasis on the role of plasma membrane protein and ATPase activity as the mechanism(s) by which alfalfa (Medicago sativa L.) cultivars exhibiting differential salt tolerance respond to NaCl environments.

OBJECTIVES

1. Determine the effect of $CaSO_4$ on ion content in alfalfa varieties under NaCl stress.

2. Characterize the effect of Ca^{2+} on plasma membrane proteins and ATPase activity in alfalfa varieties exhibiting differential NaCl tolerance.

REVIEW OF LITERATURE

Effect of Salinity on Protein Plasma Membrane

Alfalfa is among the few crop plants that have shown differential salinity tolerance and the ability to grow under high levels of salts accumulated by fertilization practices or by use of saline water for irrigation (2, 3, 67, 81). This suggests that alfalfa may possess the genetic potential for adapting to salinity, perhaps through differential ion transport across cellular membranes (84). Moreover, plasma membrane proteins may influence alfalfa's response to NaCl (13). These effects seem especially significant in view of the fact that an important aspect of plant salt tolerance is the sequestering of salts from the cytoplasm by membrane transport and compartmentation (35, 64, 65). In addition, NaCl-induced changes in electrostatic properties may influence ion transport across plasma membranes of NaCl-stressed and non-stressed tomato, Lycopersicon esculentum Mill (84). In a study on roots of barley (Hordeum vulgare L. cv CM72), Hurkman et al. (47) showed that salt stress caused increases or deceases in a number of polypeptides, but no unique polypeptides were induced by stress.

Amelioration Effect of Calcium

Calcium is an important nutrient in plants exposed to salt stress because of its role in membrane integrity and

function, cell wall extension, and cellular stress recovery (43). This suggests that supplemental calcium can mitigate the detrimental effects of high sodium concentration on growth (40). Calcium also is essential for K^+/Na^+ selectivity; markedly reducing K^+ efflux in salt-stressed plants (15).

Cramer et al. (19) investigated the effects of NaCl (0-250 $mol_c\ m^{-3}$) and Ca^{2+} (0.4 and 10 $mol_c\ m^{-3}$) on the ion activities in solution and on root growth of cotton (Gossypium hirsutum L). Ion activities in solution were analyzed using the computer program GEOCHEM. Most ion activities in a 0.1 modified Hoagland solution were significantly reduced by both NaCl and supplemental Ca^{2+}. Ion-pair formation and precipitation were significant for some ions, especially phosphate.

Cramer et al. (20) used chlorotetracycline (CTC) as a probe for membrane-associated Ca^{2+} in intact cotton root hairs indicating displacement of Ca^{2+} by Na^+ from membrane sites with increasing levels of NaCl (0 to 250 $mol_c\ m^{-3}$). They also found that K^+ (^{86}Rb) efflux increased dramatically at high salinity. An increase in external Ca^{2+} concentration (10 mol m^{-3}) mitigated both responses. Other cations and mannitol, which did not affect Ca^{2+}-CTC chelation properties, were found to have no effect on Ca^{2+}-CTC fluorescence by ethyleneglycol-N,N'-tetracetic acid (EGTA). The EGTA, which did not cross the membranes, provided an indication that reduction by Na^+ of Ca^{2+}-CTC fluorescence might be occurring primarily at the

plasma membrane.

Sodium ions can displace Ca^{2+} from the root-hair-cell membrane of cotton plants (20). These plants were grown with calcium levels of 0.4 mM and Ca^{2+}/total cation ratios of 0.37. The increased membrane Na^+ resulted in a leakage of cytosolic K^+ (^{86}Rb) ions. The specific displacement of Ca^{2+} was determined by adding CTC and measuring the amount of fluorescence resulting when CTC reacted with bound Ca^{2+}. Using similar techniques, displacement of Ca^{2+} by other ions, including Na^+, was also shown with protoplasts isolated from corn root cells (60). Other aspects of mineral nutrition, besides preserving the integrity of membranes and physiological functions, generally, illustrate the importance of adequate Ca^{2+} in the ability of plants to cope with salinity stress. Exclusion of Na^+, a feature of salt-sensitive plants like beans (55) as well as less sensitive plants like cotton (21), is an important aspect of the effective functioning of K^+/Na^+ selective mechanisms. Salinity influences anatomical features of cotton root cells, and these effects are ameliorated by additional Ca^{2+} (53).

LaHaye and Epstein (55) postulated that the Na^+/Ca^{2+} interaction takes place at the plasma membrane. These authors suggested that Na^+ acted by displacing Ca^{2+} from membranes, leading to increased membrane permeability. Additions of Ca^{2+} salts to a complete nutrient solution partly alleviated the suppression of root growth in cotton under high salinity

levels. Potassium concentrations in the root were reduced by salinity, but were restored to adequate levels by an additional supply of Ca^{2+}.

Cramer and Lauchli (18) concluded that Ca^{2+} ion activity was greatly reduced with increasing NaCl concentrations. However, this did not alter the previous basic conclusion that Na^{+} displaces Ca^{2+} from membranes (20). With a mass-action and cation-exchange relation, the latter authors supported the hypothesis that Ca^{2+} displacement occurred at different classes of membrane sites, and there appeared to be a high affinity Ca^{2+} binding site at a plasma membrane associated protein.

ATPase Systems at the Plasma Membrane

Energy for the extrusion of Na^{+} into the vacuole across the tonoplast membrane and extrusion of Na^{+} into the external medium across the plasma membrane may be provided by the proton gradients generated by these membrane H^{+}-ATPases as Na^{+}/H^{+} antiports or as Ca^{2+}-ATPase. These data support the hypothesis that increasing the activity of Ca^{2+} ions in the NaCl root media of glycophytic crop plants can reduce Na^{+} accumulation and partially reverse the growth inhibition induced by excess salinity in the rhizosphere (20, 30, 38, 54, 88). Cells of higher plants may react to a salt stress by increased vesiculation of the plasma membrane, which separates the external environment and numerous biochemical processes taking place in the cytoplasm. The energy for extrusion of Na^{+}

across the plasma membrane may be provided by proton gradients generated by the plasma membrane Ca^{2+}-ATPase.

H^+-ATPase

Katz et al. (50) reported that Na^+/H^+ antiporter played an important role in internal pH regulation in halotolerant alga Dunaliells salina L. After being exposed to saline conditions for a week, roots showed a drastic increase in growth and then decreased. They interpreted the phase of Na^+ influx as due to the activation of plasma membranes Na^+/H^+ antiporter, and the phase of Na^+ extrusion process. Furthermore, this work reported additional chemical characteristics of Na^+/K^+ antiporter. The phase of Na^+ influx was energy independent since Na influx was sensitive to uranium, an inhibitor of Na^+/K^+ antiporter, and was insensitive to vanadate. The phase of Na^+ efflux was energy-dependent where it was inhibited by vanadate. Braun et al. (12) reported the proton-transporting ATPase activity in membrane vesicles obtained from Atriplex root could be modulated by saline conditions during the growth of this halophyte. These modulations were detected from the following observations. First, when plants were grown in the absence of salt, a flat pH profile was observed, but it was transformed in the presence of salt. A high peak between pH 6.0 and 6.5 was detected. Second, kinetics of H^+-translocating activity versus ATP concentration were altered by the conditions of growth. Third,

K^+ stimulated difference in water potential formation in salt-grown, but not in non-salt-grown plants. Sze (86) reported an inhibition of H^+-translocation by K^+, rather than stimulation. Stimulation was reported in the case of radish membranes. Braun et al. (12) found that K^+ stimulation was only observed in salt-grown roots and not in the non-salt-grown plants. Finally, they concluded that the presence of NaCl was vital for proper functioning of Atriplex where K^+ stimulation and pH profile had shown optimum for ATPase activity (12).

Douglas and Walker (23) reported that the effect of salt exposure (50-100 mM NaCl) on Mg^{2+}-ATPase activity was genotype-dependent. Eredei et al. (29) reported that Plantago coronopus (a moderately salt-sensitive species), grown in a medium containing 75 and 150 mM NaCl, reduced Mg^{2+}-ATPase activity approximately 30 and 45%. Gronwald et al. (40) reported that divalent cations were most effective in activating ATPase activity, while alkali metals like K^+, Na^+, and Rb^+ provided equivalent stimulation and Li provided the least. They further indicated that tomato root plasma membrane ATPases had a pH optimum of 7.0 in the presence of Mg^{2+}-ATP. They concluded that the stimulation of ATPase activity of salt-sensitive species like corn and oats by K^+ was much greater than that by Na^+. With respect to more salt-tolerant species (Atriplex, red beet, and tomato), the ATPase activity provided equivalent stimulation (40).

CA^{2+}-ATPase

Many scientists indicated the severe effect of salinity on Ca^{2+} uptake and transport (17, 21, 24, 33, 39, 63). Cramer et al. (20) reported that increased salinity reduced the amount of Ca^{2+} bound to the plasma membrane of Gossypium hisutum L. root hairs and inhibited Ca^{2+} influx into cotton tap roots.

Suhayda et al. (84) reported that salt stress induced electrostatic changes in Lycopersicon esculentum Mill. root plasma membrane and caused surface potential to become less negative, which resulted in less cations attracted to the plasma membrane. Since Na^{+} would be the least affected cation due to its high activity in bulk solution, binding of $^{45}Ca^{2+}$ to plasma membrane would be more affected (10, 20, 60).

Other monovalent cations may also inhibit Ca^{2+} binding to the plasma membrane (Li > Cs > Rb > Na > K). Rengel (77) concluded that Na^{+} effects on the cell Ca^{2+} homostasis could be achieved either through changes in the plasma membrane fluidity, in general, and activity of Ca^{2+}-translocating channels (82) and Ca^{2+}-ATPase pumps (76) in particular sites due to very fast uptake of Na^{+} across the plasma membrane (61). The observation by Shroeder and Thuleau (82) has been refuted by Lynch and Lauchli (61) since there was no apparent change in permeability of plasma membrane after exposing Zea mays L. root protoplasts to 100 mol_c m^{-3} NaCl up to 2 h. This left the first two conclusion to further testing.

Salinity and Ion Pair Formation

Sodium, Cl^-, and SO_4^{2-} are generally the most abundant ions in sodic soils and thus are major contributors to the osmotic properties of soil solution. In addition to their effects on water potential and ionic strength of the soil solution, these ions, particularly Na^+ and Cl^-, have specific effects on structural integrity and metabolism of cells (15, 19).

The addition of NaCl causes signs of poisoning such as severe inhibition of growth, blackening of roots, necrosis, and decaying of leaves (66). Mercado and Gollek (66) found that Na salts decrease the absorption of ammonia nitrogen and as a consequence the nitrogen metabolism is manifested in an accumulation of asparagine, glutamic acid, and other amino acids. Moreover, Newton (69) and Mercado and Gollek (66) stated that Na salts disturb protein metabolism by accumulating ammonia, lysine, proline, and other amino acids, which have a toxic effect on the plant cell. With respect to carbohydrate metabolism, plants grown in NaCl increase sucrose content, contain no detectable amounts of fructose, and have a low glucose content, whereas the control plants contain mainly glucose with small amounts of sucrose and only traces of fructose (66). This apparently results from increased concentrations of a-ketoglutaric acid, but decreased concentrations of pyruvic, citric, oxalic, aconitic, and fumaric acid.

MATERIALS AND METHODS

Plant Material

Young alfalfa (Medicago sativa L.) clones of Centurion (salt-sensitive) and Condor (salt-tolerant) inoculated with salt-tolerant Rhizobium meliloti (USDA strain #1031) were grown in an aerated, hydroponic, 0.5 strength Hoagland's nutrient solution, pH 6.0 (4). Two alfalfa plants of each cultivar were grown under greenhouse conditions with a day/night regime of 14/10 h at 25/20°C (Fig. 1). After alfalfa clones were equilitbrated in a Hoagland solution for 6 days, they were exposed to 0, 88, and 132 mM of NaCl alone and mixed with $CaSO_4 \cdot H_2O$ at 7 and 14 mM in all treatment combinations. After exposing to NaCl solutions for 3, 9, and 60 d, roots were harvested and stored at -80°C.

Isolation of Plasma Membrane

The plasma membrane was isolated by the method of Giannini et al. (37). Alfalfa roots (1.5 g) were ground with a morter and pestle in a 3:1 buffer:tissue ratio (Fig. 2). The ice-cold homogenization medium contained 250 mM sucrose, 3 mM ethylenediaminetetraacetic acid (EDTA), 2 mM Na_2ATP, 10% (v/v) glycerol, 0.5% (w/v) Bovine serum albumin (BSA, fraction V powder), 0.5% (w/v) polyvinylpyrrolidone (40,000 M_r) (PVP), 2 mM phenylmethylsulfonyl fluoride (PMSF), 15 mM β-mercaptoethanol, 4 mM dithioerythreitol (DTE), 250 mMKI and

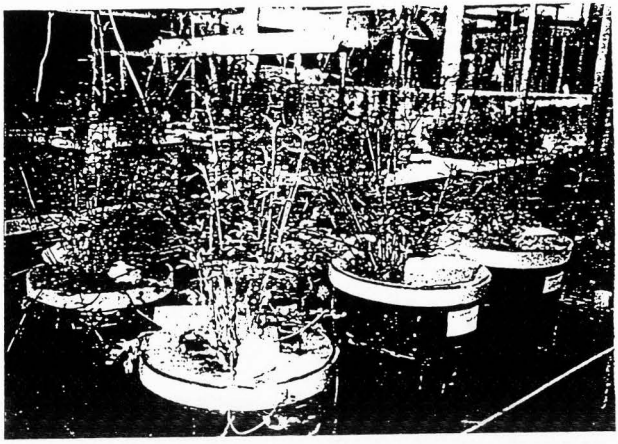

Fig. 1. Alfalfa cvs. Centurion and Condor were grown in a 0.5 strength Hoagland solution in a greenhouse.

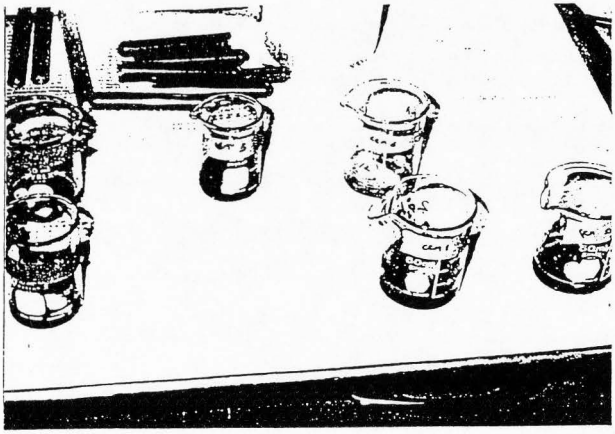

Fig. 2. Isolation of plasma membrane of both Centurion and Condor was done in a cold room of -4°C.

70 mM Tris/HCl, pH 7.7. Dithioerythreitol, PMSF, and β-mercaptoethanol were added to the medium just prior to use. The homogenate was filtered through 4 layers of cheesecloth and then centrifuged at 13,000 g for 15 min at 4°C. The 13,000-g pellet was discarded and the supernatant was centrifuged at 40,000 g (24,500 rpm) for 30 min at 4°C to obtain a microsomal pellet.

Purification of the Isolated Plasma Membrane

Further purification of the plasma membrane involved centrifugation on linear sucrose density gradients. The plasma membrane pellet was resuspended in 1.0 mL of suspension buffer containing 250 mM sucrose, 10% glycerol, 0.2% BSA, 1 mM DTE (added fresh), 1 mM PMSF (added fresh), 2 mM BTP/MES {BTP, (1,3-bis[tris(Hyroxymethyl)-methylamino]propane), MES, (2-[N-Morpholimo]ethanesaulfonic acid), pH 7.0, and gently ground in a dounce-type homogenizer and then layered onto a 36-mL discontinuous consisting of 28 ml of 45% and 8 ml of 34% (w/w) sucrose gradient (40). The sucrose solutions were buffered to pH 7.2 with 1 mM Tris/MES containing 1 mM DTE. Plasma membrane extraction loaded onto the gradient, centrifuged at 100,000 g for 2 h at 4°C in a RC 70 Sorvall ultracentrifuge (Fig. 3). The plasma membrane was collected at the 34-45% sucrose interface, quickly frozen in liquid N_2, and stored at -80°C.

Figure 3. RC 70 Sorvall.

Protein Assay

Plasma membrane protein concentration was determined by the protocol of Bradford (11) with bovine serum ablumin (BSA) as a standard. Briefly, 20 μL of solutions containing plasma membrane vesicles were each placed in separate tubes with the addition of 80 μL of ddH_2O prior to the addition of 5 mL filtered Bradford reagent. The samples were vortexed and left to sit for 10 min. Absorbance was measured at 595 nm with 6-450 UV/VIS Sargent Welch spectrophotometer.

Ohnishi Assay

The assay for ATPase activity was carried out at 37°C in a 1.0-mL volume containing 30 mM Tris/Mes (pH 6.5), 15 mM $MgSO_4$, 50 mM KCl, and 15 mM ATP (70). The reaction was initiated by the addition of 100 μL of solution (10-20 μg of plasma membrane). The solution was vortexed and placed in 37°C water bath for 20 min. The reaction was stopped by adding 5 mL of mixed reagent in 3:2:1 ratio of hydroxylamine sulfate, PVP-40 and H_2SO4:$(NH_3)Mo_7O_{24}.4H_2O$ and EDTA-Na_4:ddH_2O. After 10 min, 0.5 mL of color developer, and a combination of Na carbonate and NaOH were added prior to taking readings at 720 nm.

Elemental Assay

The root samples (500 mg) were digested in a mixture of nitric acid and perchloric acid, and concentration of Na, Ca, Mg, and K was determined using a Perkin-Elmer Model 2380 atomic absorption spectrophotometer. With respect to determination of Cl concentration, 0.1 g of dry root sample was added to 10 mL deionized water after being ground then heated to 90°C and vortexed for 10 min. The extract was titrated with 0.05 N AgNO3 (22).

Electrophoresis

The plasma membrane was removed from storage and centrifuged at 80,000 g for 1 h. The resulting pellet was suspended with a suspension buffer containg 1 mM PMSF and 1 mM

DTE added just prior to use. The plasma membrane samples, which were isolated from roots after exposing them to different salt treatments for 3, 9 and 60 days, were added with 2X treatment (containing tris, SDS, glycerol, and 2-mercaptoethanol) in equal parts prior to being run in 10% acrylamide SDS-PAGE. Vertical slab unit SE600 was used. Protein concentration was determined by the Bradford method (11) and equal amounts of protein were loaded in each well.

Silver Staining

The silver staining method was used to visualize protein bands (6). Gels were fixed in a solution containing 10% trichloroacetic acid (TCA), 5% acetic acid, and 30% methanol and shaken for 30 min prior to a second fixation in a solution containing 25% glutaraldehyde. After 30 min on a shaker, the gels were rinsed and returned to the shaker for 10 min followed by an overnight rinse in a 10% ethanol. Gels were shaken for 13 min in a diamine solution containing 19.4% $AgNO_3$, 0.36% NaOH, concentrated NH_3OH, and ethanol. After being shaken in deionized water for 5 min, the reaction was initiated by adding 0.005% citric acid, 0.0185% formaldehyde, and 10% ethanol. The reaction was stopped with the addition of a solution containing 10% acetic acid.

RESULTS AND DISCUSSIONS

Sodium Chloride and Calcium Sulfate Induced Protein Differences in Alfalfa (Medicago sativa L.)

Salt treatments induced changes in the concentration of protein bands (Figs. 4, 5, 6, and 7) regardless of whether the proteins were extracted from Centurion or Condor. Increases or decreases may depend on different concentrations of NaCl and $CaSO_4$ (4, 47). Subtle changes in protein bands were difficult to obtain and were detected on high resolution 2D gels. Problems caused by the presence of nonprotein components interfere with resolution of proteins (46).

In Fig. 4, salt treatments showed terminate bands of 251 kD and 190 kD when alfalfa roots were exposed to 88 mM NaCl and the interaction of 7 mM $CaSO_4$ and 132 mM NaCl, respectively. Salt treatments induced 154 kD and 275 kD when alfalfa roots were exposed to the interaction of 7 mM $CaSO_4$ and 88 mM NaCl and the interaction of 14 mM $CaSO_4$ and 132 mM NaCl, respectively. There was a terminate of polypeptide of 29 kD when the Centurion cultivar was exposed to 7 mM $CaSO_4$ and 132 mM NaCl for 6 days (Fig. 5). With respect to the Condor cultivar, inducement of polypetides (36 kD) was noticed when roots were exposed to the interaction of 7 mM $CaSO_4$ and 132 mM NaCl and 14 mM $CaSO_4$ for 3 days (Fig. 6). Terminate polypeptides (20.1 kD) occurred when the same cultivar was

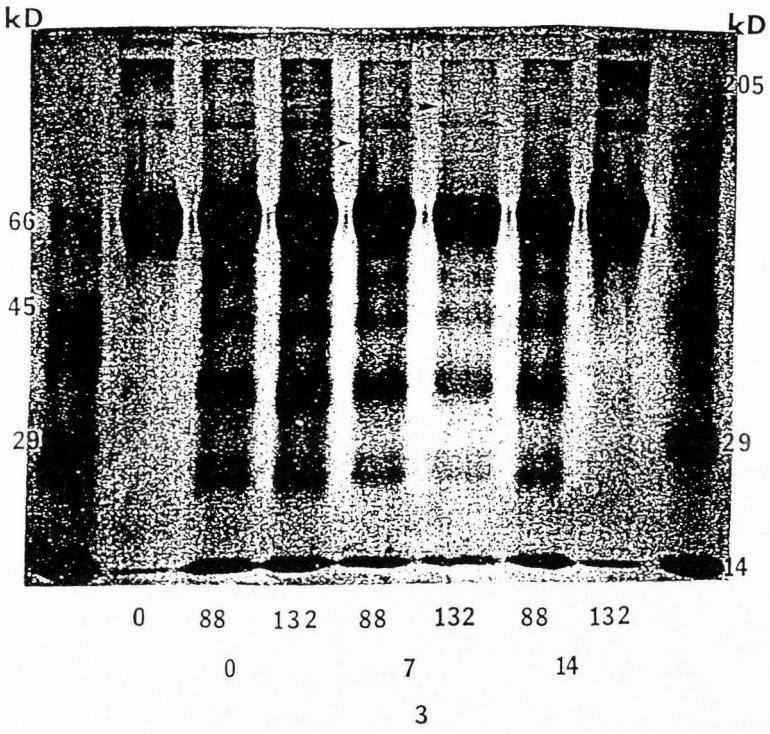

Fig. 4. The effects of NaCl and the interaction of NaCl and $CaSO_4$ on alfalfa, Medicago sativa L. cv Centurion, root plasma membrane proteins separated on 10% acrylamide SDS-PAGE and silver-stained. The position of protein standards of known molecular wewights (kDa) are pointed out by the margins. NaCl-induced proteins are indicated by arrows. NcCl-terminated proteins are indicated by double arrows. The first, second, and third rows of numbers at the bottom of figure indicate NaCl level (mM), $CaSO_4$ level (mM), and length of exposure (d), respectively.

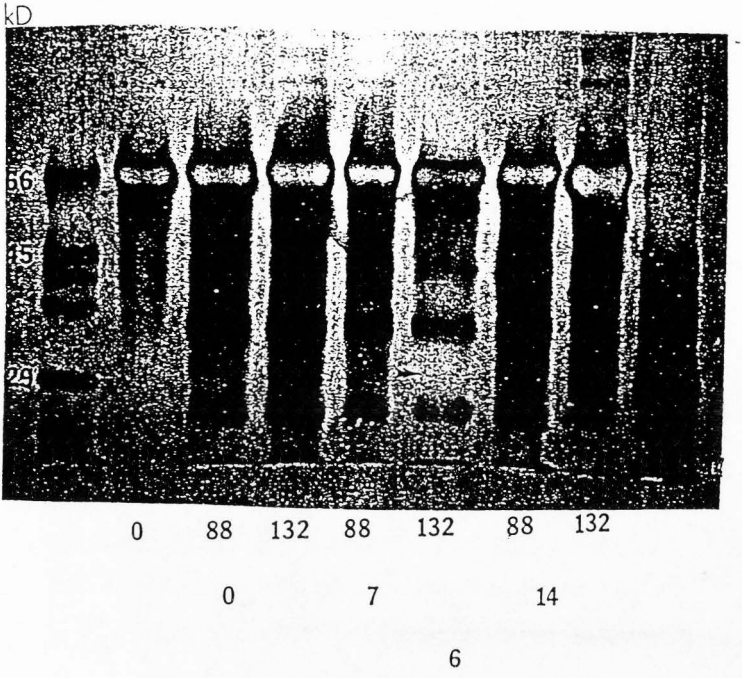

Fig. 5. The effects of NaCl and the interaction of NaCl and $CaSO_4$ on alfalfa, Medicago sativa L. cv Centurion, root plasma membrane proteins separated on 10% acrylamide SDS-PAGE and silver-stained. The position of protein standards of known molecular wewights (kDa) are pointed out by the margins. NaCl-induced proteins are indicated by arrows. NcCl-terminated proteins are indicated by double arrows. The first, second, and third rows of numbers at the bottom of figure indicate NaCl level (mM), $CaSO_4$ level (mM), and length of exposure (d), respectively.

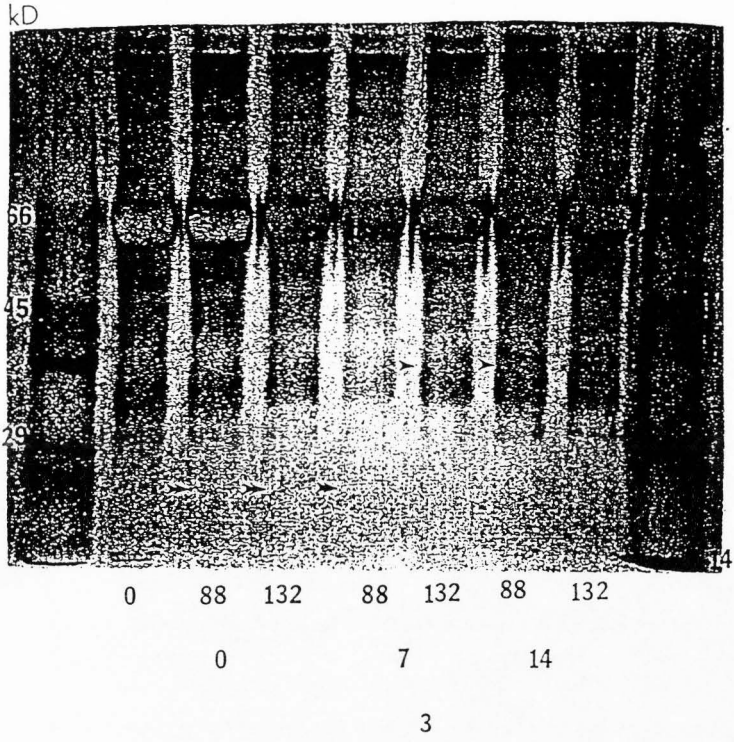

Fig. 6. The effects of NaCl and the interaction of NaCl and $CaSO_4$ on alfalfa, Medicago sativa L. cv Condor, root plasma membrane proteins separated on 10% acrylamide SDS-PAGE and silver-stained. The position of protein standards of known molecular wewights (kDa) are pointed out by the margins. NaCl-induced proteins are indicated by arrows. NcCl-terminated proteins are indicated by double arrows. The first, second, and third rows of numbers at the bottom of figure indicate NaCl level (mM), $CaSO_4$ level (mM), and length of exposure (d), respectively.

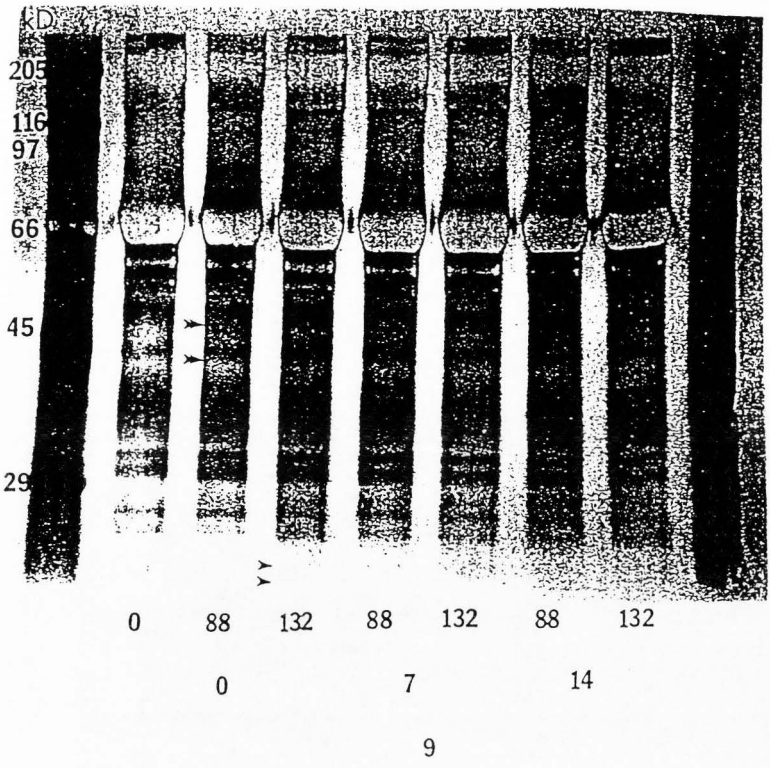

Fig. 7. The effects of NaCl and the interaction of NaCl and $CaSO_4$ on alfalfa, Medicago sativa L. cv Condor, root plasma membrane proteins separated on 10% acrylamide SDS-PAGE and silver-stained. The position of protein standards of known molecular wewights (kDa) are pointed out by the margins. NaCl-induced proteins are indicated by arrows. NcCl-terminated proteins are indicated by double arrows. The first, second, and third rows of numbers at the bottom of figure indicate NaCl level (mM), $CaSO_4$ level (mM), and length of exposure (d), respectively.

exposed to salts without $CaSO_4$ (Fig. 6). Figure 7 shows polypeptide inducement (17 kD and 14 kD) when Condor was exposed to 132 mM NaCl for 9 days. It was surprising not to find any terminate or induced polypeptides of lower molecular weights as cited (16). Only Al-Neimi et al. (4) showed inducement of 208 kD in the roots of cultivar Mecca. These results might be due to the detrimental effect of salts on cells where ankyrin, spectrins, or actin (serving as anchors for cytoskeletal proteins, which are a network of fibrous proteins constructed beneath the surface membrane) (36) were left attached to the plasma membrane during isolation of plasma membranes. This observation might explain the appearance of polypeptides (Figs. 4, 5, 6, and 7). Another explanation might be due to detrimental effect of salt on the cytoskeleton, leaving the organelles (mitochondria, tonoplast, and Golgi apparatus) attached to proteins of plasma membrane by the calcium ions, i.e., 275 kD. This observation was supported by many articles where plasma membrane extraction was contaminated with mitochondria, tonoplast, and Golgi apparatus (5, 9, 37, 72, 74). Thus, inhibitors (KCl, Na_2VO_3, KNO_3) of the contaminants (mitochondria and tonoplast) were used to measure the ATPase activity to determine the purity of plasma membrane extraction. Na_2VO_3 and NaN_3 inhibited plasma membrane and mitochondria, respectively.

The results shown in Tables 1 through 4 reflected the different responses of ATPase enzyme extracted from alfalfa

Table 1. Effect of inhibitors on alfalfa, cv Centurion, plasma membrane protein exposed to different salt treatments for 3 days.

	ATPase activity (μmol Pi/mg.h)		
Salt treatments	Control	Na_2VO_3	NaN_3
Control	20.6	0.0	18.0
88 mM NaCl	21.7	4.7	12.7
132 mM NaCl	8.6	0.0	9.3
7 mM $CaSO_4$ 88 mM NaCl	9.3	0.0	9.3
7 mM $CaSO_4$ 132 mM NaCl	13.9	0.0	13.9
14 mM $CaSO_4$ 88 mM NaCl	18.3	11.6	6.9
14 mM $CaSO_4$ 132 mM NaCl	15.3	4.7	13.9

Values indicated are means of three replications. Isolation of plasama membrane for a particular cultivar was done twice, but the determination of ATPase activity was tripled.

Table 2. Effect of inhibitors on alfalfa, cv Centurion, plasma membrane protein exposed to different salt treatment for 9 days.

Salt treatment	ATPase activity (μmol Pi/mg.h) Control	Na_2VO_3	NaN_3
Control	7.0	3.1	9.3
88 mM NaCl	7.0	0.0	4.7
132 mM NaCl	4.7	3.1	9.3
7 mM $CaSO_4$ 88 mM NaCl	7.0	0.0	4.7
7 mM $CaSO_4$ 132 mM NaCl	9.3	4.7	4.7
14 mM $CaSO_4$ 88 mM NaCl	9.3	7.0	3.1
14 mM $CaSO_4$ 132 mM NaCl	9.3	0.0	3.1

Values indicated are means of three replications. Isolation of plasama membrane for a particular cultivar was done twice, but the determination of ATPase activity was tripled.

Table 3. Effect of inhibitors on alfalfa, cv Condor, plasma membrane protein exposed to different salt treatments for 3 days.

	ATPase activity (μmol Pi/mg.h)		
Salt treatment	Control	Na_2VO_3	NaN_3
Control	13.9	7.9	13.9
88 mM NaCl	10.2	0.0	10.5
132 mM NaCl	9.3	0.0	7.9
7 mM $CaSO_4$ 88 mM NaCl	13.9	0.0	7.9
7 mM $CaSO_4$ 132 mM NaCl	9.3	0.0	7.9
14 mM $CaSO_4$ 88 mM NaCl	12.7	7.9	9.3
14 mM $CaSO_4$ 132 mM NaCl	12.5	0.0	9.3

Values indicated are means of three replications. Isolation of plasama membrane for a particular cultivar was done twice, but the determination of ATPase activity was tripled.

Table 4. Effect of inhibitors on alfalfa, cv Condor, plasma membrane protein exposed to different salt treatments for 9 days.

Salt treatments	ATPase activity (μmol Pi/mg.h) Control	Na_2VO_3	NaN_3
Control	20.2	0.0	13.9
88 mM NaCl	9.3	0.0	13.9
132 mM NaCl	13.9	0.0	13.9
7 mM $CaSO_4$ 88 mM NaCl	23.1	0.0	12.7
7 mM $CaSO_4$ 132 mM NaCl	31.9	0.0	20.2
14 mM $CaSO_4$ 88 mM NaCl	20.3	0.0	19.3
14 mM $CaSO_4$ 132 mM NaCl	23.1	0.0	8.7

Values indicated are means of three replications. Isolation of plasama membrane for a particular cultivar was done twice, but the determination of ATPase activity was tripled.

cvs Centurion and Condor that were exposed to different salt treatments for different times of exposure. The ATPase enzyme, which was not exposed to salt treatments, in general, had the highest value.

In Table 1, the ATPase activity increased 4.85%, then decreased 5.1% when Centurion roots were exposed to 88 mM NaCl and 132 mM NaCl, respectively. The addition of $CaSO_4$ increased ATPase activity by 7.5%, 38.1%, 52.9%, and 43.8% when Centurion was exposed to 7 mM $CaSO_4$ and 88 mM NaCl; 7 mM $CaSO_4$ and 132 mM NaCl; 14 mM $CaSO_4$ and 88 mM NaCl; and 14 mM $CaSO_4$ and 132 mM NaCl, repectively.

Exposure of Centurion to 88 mM NaCl did not change the ATPase activity, but exposure to 132 mM NaCl decreased ATPase activity by 48.9%. The addition of $CaSO_4$ ameliorated the effect of salt stress by increasing ATPase activity to its normal value of that of the control. ATPase activity increased by 49.5% when Centurion was exposed to 7 mM $CaSO_4$ and 132 mM NaCl; 14 mM $CaSO_4$ and 88 mM NaCl; and 14 mM $CaSO_4$ and 132 mM NaCl (Table 2).

Results shown in Table 3 were contrary to results obtained in Table 1. There was a gradual decrease in ATPase activity by 36.3% and 49.5% when Condor was exposed to 88 mM NaCl and 132 mM NaCl, respectively. Addition of $CaSO_4$ increased ATPase activity to values higher than the one obtained when Condor was exposed to 88 mM NaCl and 132 mM

NaCl. It can be concluded that ATPase activity increased by 26.7% when Condor was exposed to 14 mM $CaSO_4$ and 88 mM NaCl, and to 14 mM $CaSO_4$ and 132 mM NaCl.

Results shown in Table 4, in general, were highest when compared to data in previous Tables 1 through 3. In the abscence of $CaSO_4$, ATPase activity decreased and increased when Condor was exposed to 88 mM NaCl and 132 mM NaCl, respectively. However, in the presence of $CaSO_4$, the values obtained were higher than the one obtained when Condor was exposed to 88 mM NaCl. ATPase activity increased 59.7%, 70.8%, 54.2%, and 59.7% when Condor was exposed to 7 mM $CaSO_4$ and 88 mM NaCl; 7 mM $CaSO_4$ and 132 mM NaCl; 14 mM $CaSO_4$ and 88 mM NaCl; and 14 mM $CaSO_4$ and 132 mM NaCl, respectively.

Time of exposure to different salt treatment affected the ATPase activity, but this effectiveness depended on the cultivar. It seemed that for a long exposure to salt treatment, Centurion (a salt-sensitive cultivar) ATPase activity was decreased. Alternatively, long exposure to salt treatments increased ATPase activity of Condor, the salt-tolerant cultivar.

Upon exposing alfalfa cultivars to NaCl and $CaSO_4$, ATPase activity was decreased, then increased. This reduction might be explained by open interpretations. For instance, salt stress might impair the catalytic efficiency of the enzyme or through its effects on the lipid composition of the membrane (40). It was well-established that the salt-stress might alter

the lipid composition of membranes in plants (52). This modulation of ATPase activity was an indirect result of the effects of NaCl on plasma membrane lipid composition. For instance, certain plant species showed an increase in the free-sterol content (52) under NaCl-stress. Also, it showed an increase of sterol/phospholipid ratio of microsomal membrane (23). These changes were considered to have adaptive significance because they might decrease passive permeability to salt and increase membrane stability (52). Alternatively, the exposure of plants to NaCl resulted in the appearance of isozymic activity that enhanced the ability of plant cells to utilize metabolic energy more efficiently (78).

The greater affinity of the plasma membrane ATPase for ATP might be the result of gene-encoding isozymes (80). Isogenes of the plasma membrane ATPase were identified in Arabidopsis thaliana (44), Nicotiana plumbaginitolia (73), and tomato Lycopersicon esculentum Mill. (31). However, there were reports of NaCl-induced changes in many properties of enzymes (14, 48, 49, 79). Examples of changes in enzyme properties that were induced by external stress were reported for LDH and ADH (78). Therefore, in order to survive under salt stress, cells seemed to express a set of isozymes that exhibit a higher affinity towards ATP. These isozymes were assumed to maintain cellular metabolism under stress conditions where supply of energy was limited (32, 42, 45). These cells appeared to have the ability to express new enzyme

activity and to control metabolic processes such as the vacuolar pH maintenance (79) and plasma membrane proton transport (78), under conditions of increased energy demand and reduced ATP turnover caused by NaCl-stress. The increase in ATPase activity suggested the modulation of certain properties of this enzyme, such as the affinity for their respective substrates. It was assumed that these isozymes might increase the uptake and transport of K^+ (20, 21, 39, 68, 83) upon addition of Ca^{2+}, but this last observation needed further research.

Amelioration Effect of $CaSO_4$ on Ion Accumulation in Alfalfa under Salt Stress

Na^+

The Na^+ concentrations in roots (Fig. 8) were higher when the Centurion cultivar was exposed to 88 mM and 132 mM NaCl than when exposed to Ca^{2+} (7 mM and 14 mM). Addition of $CaSO_4$ to the medium alleviated the detrimental effects of NaCl on roots. When Centurion roots were exposed to salt treatments, Na^+ concentrations were lower in 9-day salt treatments than in either 3-day or 60-day.

The same results were achieved when considering Na^+ concentration in the Condor cultivar (Fig. 9). There was only one difference between the last two figures (8 and 9). The Na^+ concentration in Condor roots was lower than 4 meq/L, while Na^+ concentration in Centurion exceeded 11 meq/L.

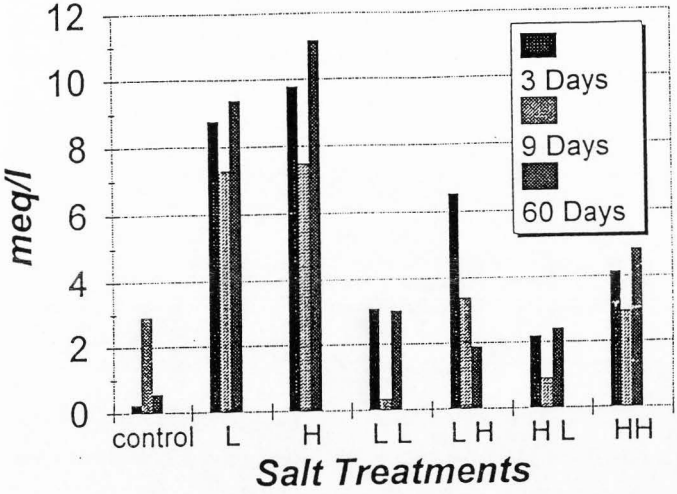

Fig. 8. Effect of salt treatments on Centurion Na^+ content in roots (P = 0.01) exposed to 3, 9, and 60 days. The first three columns are without $CaSO_4$ (control; L = 88 mM NaCl; H = 132 mM NaCl). The rest of the columns are with $CaSO_4$ (LL = 7 mM $CaSO_4$ + 88 mM NaCl; LH = 7 mM $CaSO_4$ + 132 mM NaCl; HL = 14 mM $CaSO_4$ + 132 mM NaCl). (See analysis of variance in Table 6, Appendix E.)

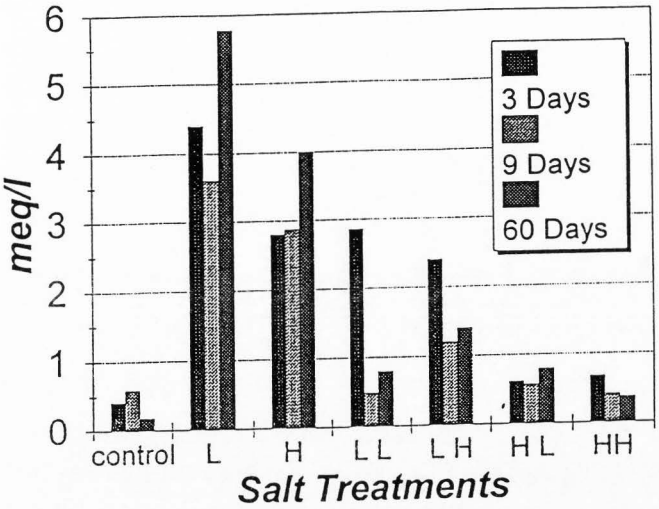

Fig. 9. Effect of salt treatments on Condor Na^+ content in roots (P = 0.01) exposed to 3, 9, and 60 days. The first three columns are without $CaSO_4$ (control; L = 88 mM NaCl; H = 132 mM NaCl). The rest of the columns are with $CaSO_4$ (LL = 7 mM $CaSO_4$ + 88 mM NaCl; LH = 7 mM $CaSO_4$ + 132 mM NaCl; HL = 14 mM $CaSO_4$ + 132 mM NaCl). (See analysis of variance in Table 6, Appendix E.)

Ca^{2+}

The Ca^{2+} levels were low when Centurion roots had been exposed to 88 mM and 132 mM NaCl (Fig. 10). This was a normal response since only roots extracted Ca^{2+} from the medium. It was apparent that Ca^{2+} levels were high when alfalfa roots were exposed to 7 mM and 14 mM $CaSO_4$. Moreover, it was noted that when roots were exposed to 132 mM NaCl, in the presence of $CaSO_4$, Ca^{2+} levels were lower than roots exposed to 88 mM NaCl. Ca^{2+} levels in roots exposed for 9 days were lower than those exposed for 3 days and 60 days. Condor roots responded in a way similar to the way Centurion responded (Fig. 11). When $CaSO_4$ had been added to the medium, Ca^{2+} levels were high. However, high levels of NaCl decreased the concentration of Ca^{2+} in roots. In general, Na^+ accumulations were similar in both Centurion and Condor.

K^+

The K^+ levels of Condor were much lower than those of Centurion (Figs. 12 and 13). The highest level of K^+ reached in Condor was 0.7 meq/L, whereas the highest level in Centurion was 8 meq/L. Each of the cultivars responded differently when exposed to different salt treatments for different times. For instance, in the presence of salt treatments, Centurion exposed for 9 days showed tremendous decrease in K^+ levels. On the other hand, Condor roots, which were exposed to 88 mM NaCl and 132 mM NaCl for 9 days, resulted in the highest K^+ levels. In the presence of $CaSO_4$,

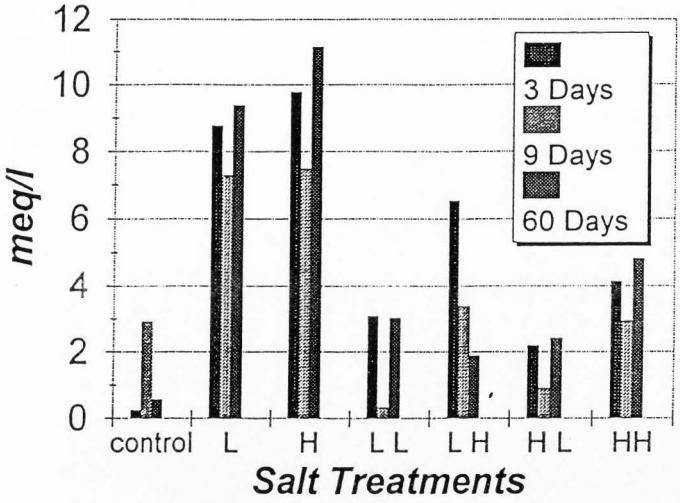

Fig. 10. Effect of salt treatments on Centurion Ca^{2+} content in roots (P = 0.01) exposed for 3, 9, and 60 days. The first three columns are without $CaSO_4$ (control; L = 88 mM NaCl; H = 132 mM NaCl). The rest of the columns are with $CaSO_4$ (LL = 7 mM $CaSO_4$ + 88 mM NaCl; LH = 7 mM $CaSO_4$ + 132 mM NaCl; HL = 14 mM $CaSO_4$ + 88 mM NaCl; HH = 14 mM $CaSO_4$ + 132 mM NaCl). (See analysis of variance in Table 7, Appendix E.)

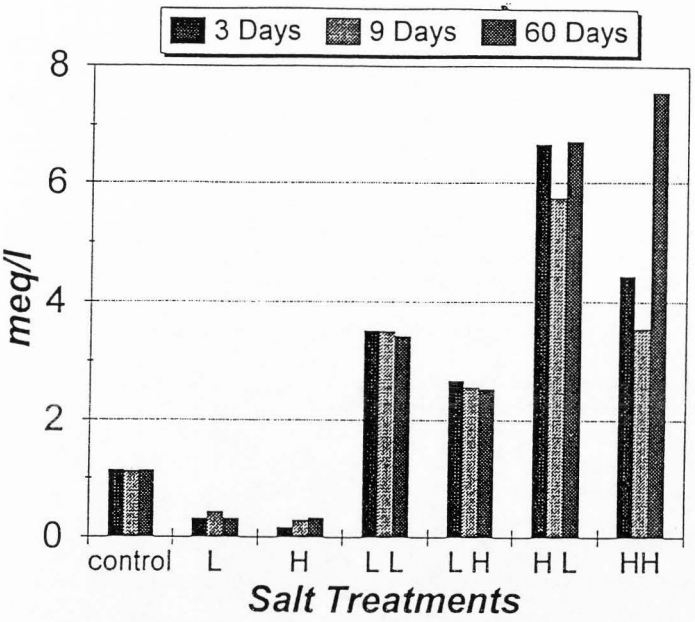

Fig. 11. Effect of salt treatments on Condor Ca^{2+} content in roots (P = 0.01) exposed for 3, 9, and 60 days. The first three columns are without $CaSO_4$ (control; L = 88 mM NaCl; H = 132 mM NaCl). The rest of the columns are with $CaSO_4$ (LL = 7 mM $CaSO_4$ + 88 mM NaCl; LH = 7 mM $CaSO_4$ + 132 mM NaCl; HL = 14 mM $CaSO_4$ + 88 mM NaCl; HH = 14 mM $CaSO_4$ + 132 mM NaCl). (See analysis of variance in Table 7, Appendix E.)

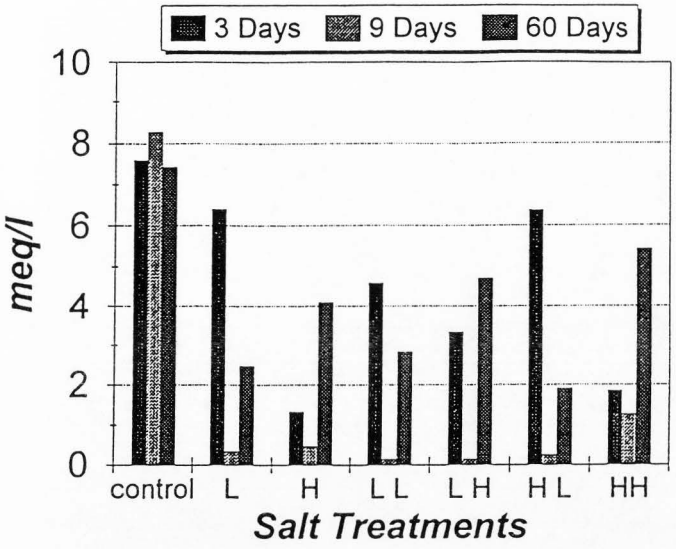

Fig. 12. Effect of salt treatments on Centurion K^+ content in roots ($P = 0.01$) exposed for 3, 9, and 60 days. The first three columns are without $CaSO_4$ (control; L = 88 mM NaCl; H = 132 mM NaCl). The rest of the columns are with $CaSO_4$ (LL = 7 mM $CaSO_4$ + 88 mM NaCl; LH = 7 mM $CaSO_4$ + 132 mM NaCl; HL = 14 mM $CaSO_4$ + 88 mM NaCl; HH = 14 mM $CaSO_4$ + 132 mM NaCl). (See analysis of variance in Table 8, Appendix E.)

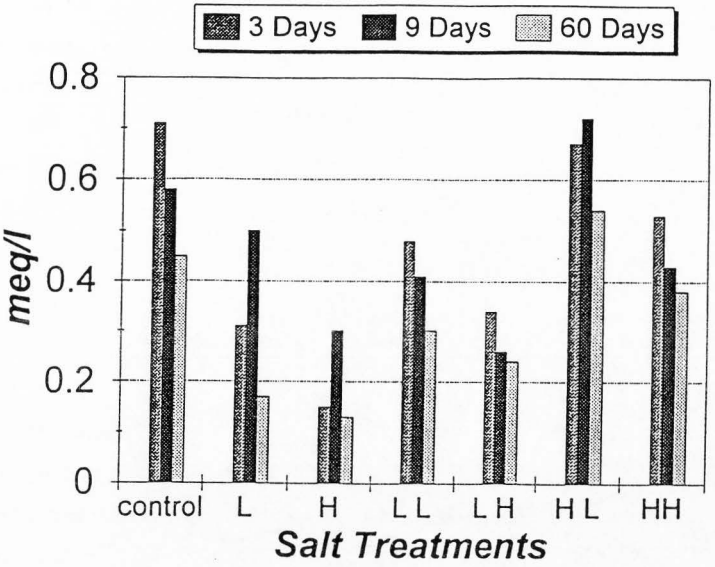

Fig. 13. Effect of salt treatments on Condor K^+ content in roots (P = 0.01) exposed for 3, 9, and 60 days. The first three columns are without $CaSO_4$ (control; L = 88 mM NaCl; H = 132 mM NaCl). The rest of the columns are with $CaSO_4$ (LL = 7 mM $CaSO_4$ + 88 mM NaCl; LH = 7 mM $CaSO_4$ + 132 mM NaCl; HL = 14 mM $CaSO_4$ + 88 mM NaCl; HH = 14 mM $CaSO_4$ + 132 mM NaCl). (See analysis of variance in Table 8, Appendix E.)

K^+ levels decreased directly proportional to the increasing days of exposure. The addition of 14 mM $CaSO_4$ improved K^+ levels in Centurion when exposed to 88 mM and 132 mM NaCl. Moreover, when Centurion roots were exposed to 88 mM NaCl, the interaction of 88 mM NaCl and 7 mM $CaSO_4$ showed the same pattern. To be more precise, K^+ levels from 60-day exposures were lower than those of 3 days. By contrast, measurements of K^+ levels from 132 mM NaCl treatment, interaction between 7 mM $CaSO_4$ and 132 mM NaCl, and the interaction between 14 mM $CaSO_4$ and 132 mM NaCl all showed similar patterns.

Mg^{2+}

Both results obtained from Centurion and Condor (Figs. 14 and 15) showed the same Mg^{2+} pattern. The lowest levels of Mg^{2+} were achieved by exposing Centurion and Condor for 9 days, while the highest levels were obtained by exposing Centurion and Condor to 3 days. A reduction in Mg^{2+} levels resulted when exposed to only NaCl, which was followed by an increase in Mg^{2+} when $CaSO_4$ levels were added.

K^+/Na^+

It was apparent that K^+/Na^+ ratio decreased and increased when both alfalfa cultivars (Figs. 16 and 17) were exposed to salt levels without $CaSO_4$ and with $CaSO_4$, respectively. At high $CaSO^4$ levels (14 mM), K^+/Na^+ ratio increased, which means that there was a relief in K^+ concentrations. These observations were similar in both Condor and Centurion, but the main

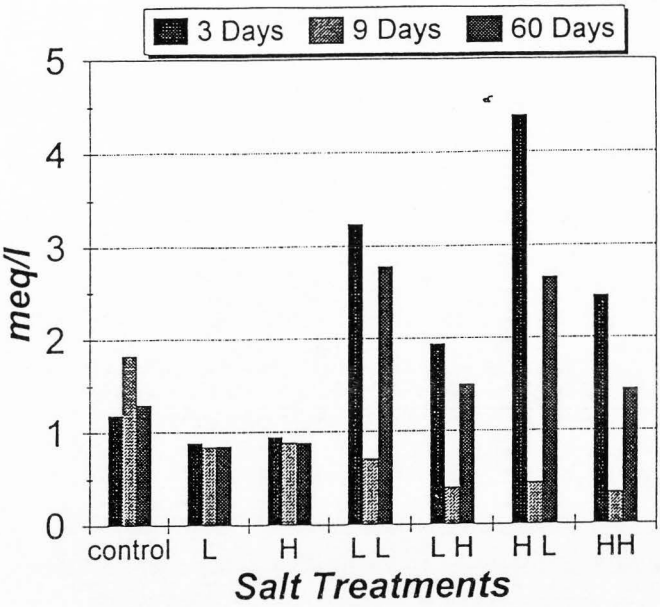

Fig. 14. Effect of salt treatments on Centurion Mg^{2+} content in roots (P = 0.01) exposed for 3, 9, and 60 days. The first three columns are without $CaSO_4$ (control; L = 88 mM NaCl; H = 132 mM NaCl). The rest of the columns are with $CaSO_4$ (LL = 7 mM $CaSO_4$ + 88 mM NaCl; LH = 7 mM $CaSO_4$ + 132 mM NaCl; HL = 14 mM $CaSO_4$ + 88 mM NaCl; HH = 14 mM $CaSO_4$ + 132 mM NaCl). (See analysis of variance in Table 9, Appendix E.)

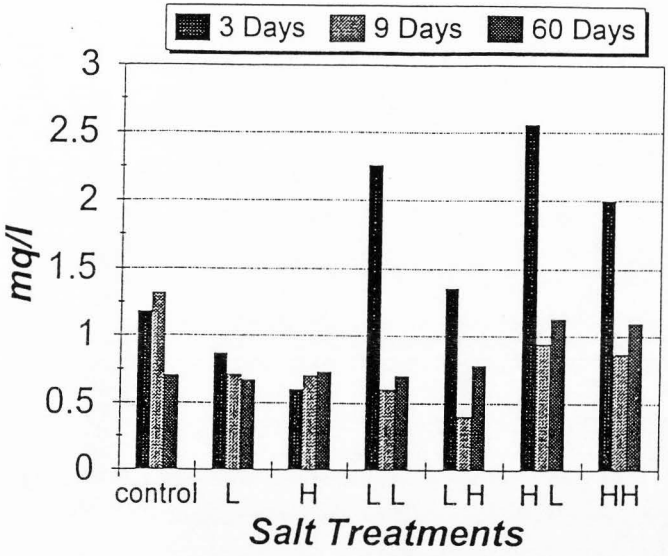

Fig. 15. Effect of salt treatments on Condor Mg^{2+} content in roots (P = 0.01) exposed for 3, 9, and 60 days. The first three columns are without $CaSO_4$ (control; L = 88 mM NaCl; H = 132 mM NaCl). The rest of the columns are with $CaSO_4$ (LL = 7 mM $CaSO_4$ + 88 mM NaCl; LH = 7 mM $CaSO_4$ + 132 mM NaCl; HL = 14 mM $CaSO_4$ + 88 mM NaCl; HH = 14 mM $CaSO_4$ + 132 mM NaCl). (See analysis of variance in Table 9, Appendix E.)

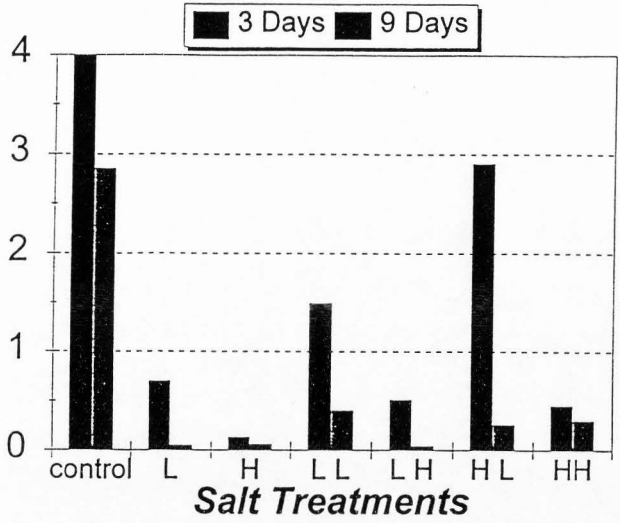

Fig. 16. Effect of salt treatments on Centurion K^+/Na^+ content in roots (P = 0.01) exposed for 3, 9, and 60 days. The first three columns are without $CaSO_4$ (control; L = 88 mM NaCl; H = 132 mM NaCl). The rest of the columns are with $CaSO_4$ (LL = 7 mM $CaSO_4$ + 88 mM NaCl; LH = 7 mM $CaSO_4$ + 132 mM NaCl; HL = 14 mM $CaSO_4$ + 88 mM NaCl; HH = 14 mM $CaSO_4$ + 132 mM NaCl). (See analysis of variance in Table 10, Appendix E.)

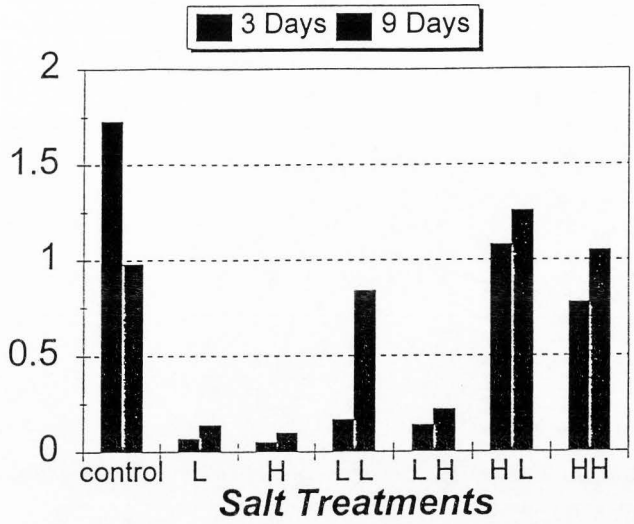

Fig. 17. Effect of salt treatments on Condor K^+/Na^+ content in roots (P = 0.01) exposed for 3, 9, and 60 days. The first three columns are without $CaSO_4$ (control; L = 88 mM NaCl; H = 132 mM NaCl). The rest of the columns are with $CaSO_4$ (LL = 7 mM $CaSO_4$ + 88 mM NaCl; LH = 7 mM $CaSO_4$ + 132 mM NaCl; HL = 14 mM $CaSO_4$ + 88 mM NaCl; HH = 14 mM $CaSO_4$ + 132 mM NaCl). (See analysis of variance in Table 10, Appendix E.)

difference was in the pattern of the results obtained. Time exposure altered the pattern. Results obtained after 3 days of exposure were lower than those after 9 days (Fig. 17). The K^+/Na^+ ratio in Centurion showed an opposite pattern where results obtained after 3 days were higher than those after 9 days (Fig. 16).

Ca^{2+}/Na^+

The addition of $CaSO_4$ affected significantly the balance of Ca^{2+} ions and Na^+ ions by increasing the Ca^{2+}/Na^+ ratio (Figs. 18 and 19). Both figures showed the same pattern. First, there was a decrease in Ca^{2+}/Na^+ due to the absence of $CaSO_4$ and the presence of NaCl. Second, an increase of Ca^{2+}/Na^+ ratio was apparent since the addition of $CaSO_4$ had been made to the medium. On the other hand, results obtained after 3 days of exposure were lower than after 9 days (Figs. 18 and 19).

In conclusion, with salt treatments, alfalfa cultivars responded differently in ion accumulation after being exposed for 3 and 9 days. It was apparent that the K^+/Na^+ status was severely affected by salt stress, but it was ameliorated by the addition of $CaSO_4$.

Nonhalophytes, plants sensitive to saline environments, suffer from various metabolic disorders, and from inhibition of growth and development when subjected to saline conditions (56). It was well stated in related articles that high Na^+ concentration can cause disturbances in calcium nutrition (63). On the other hand, supplemental Ca^{2+} can mitigate the

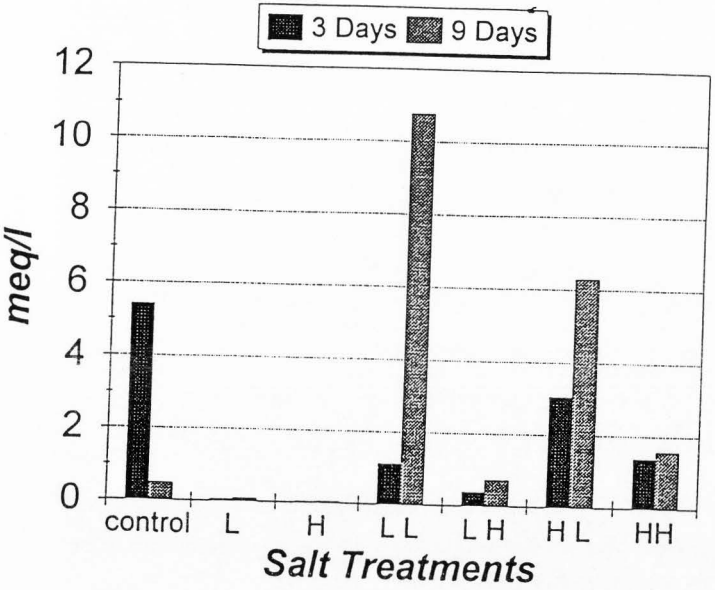

Fig. 18. Effect of salt treatments on Centurion Ca^{2+}/Na^{+} ratio in roots (P = 0.01) exposed for 3 and 9 days. The first three columns are without $CaSO_4$ (control; L = 88 mM NaCl; H = 132 mM NaCl). The rest of the columns are with $CaSO_4$ (LL = 7 mM $CaSO_4$ + 88 mM NaCl; LH = 7 mM $CaSO_4$ + 132 mM NaCl; HL = 14 mM $CaSO_4$ + 88 mM NaCl; HH = 14 mM $CaSO_4$ + 132 mM NaCl). (See analysis of variance in Table 11, Appendix E.)

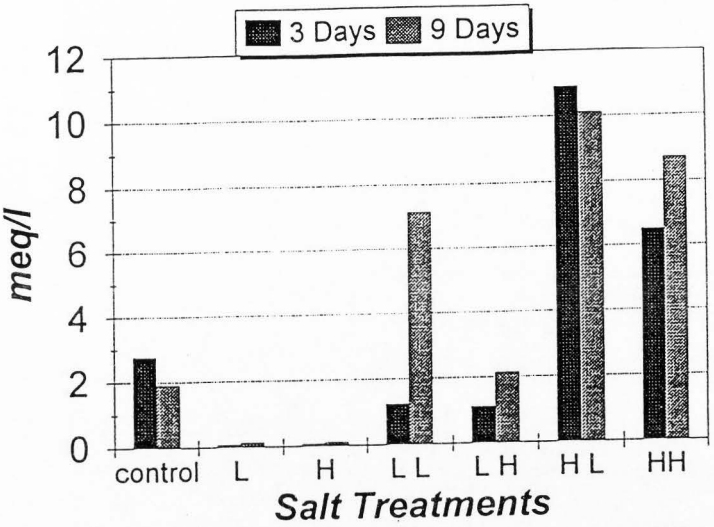

Fig. 19. Effect of salt treatments on Condor Ca^{2+}/Na^{+} ratio in roots (P = 0.01) exposed for 3 and 9 days. The first three columns are without $CaSO_4$ (control; L = 88 mM NaCl; H = 132 mM NaCl). The rest of the columns are with $CaSO_4$ (LL = 7 mM $CaSO_4$ + 88 mM NaCl; LH = 7 mM $CaSO_4$ + 132 mM NaCl; HL = 14 mM $CaSO_4$ + 88 mM NaCl; HH = 14 mM $CaSO_4$ + 132 mM NaCl). (See analysis of variance in Table 11, Appendix E.)

detrimental effects of high Na^+ on growth (19, 54), but inadequate Ca^{2+} concentrations at high Na^+ adversely affect growth and function of membranes (57). However, Ca^{2+} exerts a protective effect on cells by maintaining adequate K^+ status and K^+/Na^+ selectivity, and inhibiting Na^+ influx in alfalfa cultivars under saline conditions (21, 27, 38). Increasing concentrations of NaCl significantly inhibited K^+ influx (Figs. 12 and 13). High external levels of Ca^{2+} clearly decreased the permeability to K^+ of plasma membranes in alfalfa roots and prevented the leakage of K^+ from root cells, thus maintaining a high intracellular concentration of K^+ in roots under high NaCl stress. These results were consistent to what Kent and Lauchli (51), Van Steveninck (87), Enoch and Glinka (26), Quintero and Hanson (75), Cramer et al. (20), and Oka et al. (71) had observed. They ascribed the increased efflux of K^+ to displacement of Ca^{2+} from the plasma membranes of the root cells by external Na^+. They also ascribed that high external levels of Ca^{2+} compensated for the displacement of membrane-associated Ca^{2+} by Na^+ and minimized the leakage of K^+ from the root cells. The displacement of membrane-associated Ca^{2+} by external Na^+ was related to the ratio of ion activities of Ca^{2+} and Na^+, $(Ca^{2+})/(Na^+)^2$, in the external medium (8, 19).

The decrease in root Ca^{2+} level at low Ca^{2+}/Na^+ ratios was significantly greater in the salt-sensitive genotype (Centurion) than in the salt-tolerant one (Condor). Similar

observations were made by Elzam and Epstein (25) in Agropyron elongatum and A. intermedium where the differences in their tolerance for salinity (NaCl) were associated with differences in their calcium uptake. Condor cultivar was better able than Centurion to exclude Na^+ from its roots and to maintain higher K^+/Na^+ ratios when exposed to high levels of $CaSO_4$.

Many proteins may have been either discarded throughout the isolation steps of plasma membrane or degraded by proteases (34). This may account for the appearance of small molecular weights not being observed during electrophoresis despite the fact that relatively high levels of ions were obtained from the roots. Alternatively, it may lead us to conclude that salinity caused some plasma membrane perturbation by ionic strength, which affects Ca displacement or specific effects on membrane receptors (1). Supplemental calcium, however, ameliorated the effects of salinity by increasing Ca entry and, thus, improving K and Mg status while reducing Na entry.

SUMMARY AND CONCLUSIONS

Summary

A greenhouse study was conducted to investigate the effect of $CaSO_4$ and NaCl on plasma membrane protein and ion accumulation of two alfalfa cultivars: Centurion, which was considered to be salt-sensitive, and Condor, which was considered to be salt-tolerant.

Plants were grown in an aerated hydroponic 0.5 strength Hoagland's nutrient solution, pH 6.0. After 3, 9, and 60 days, alfalfa roots was harvested. Part of the harvested roots was used for isolating plasma membrane protein, whereas the other part was dried for determining Na^+, Ca^{2+}, K^+, and Mg^{2+} levels.

Following the procedure of Giannini et al. (37) for isolating plasma membranes, chemical tests were conducted to confirm that the plasma membranes in question were highly purified. These tests were electrophoresis and the Ohnishi assay. The results are summaried as follows:

1. Salt treatments terminated the synthesis of 251 kD and 190 kD polypeptides when alfalfa roots were exposed to 88 mM NaCl and the interaction of 7 mM $CaSO_4$ and 132 mM NaCl, respectively (Fig. 4).

2. Salt treatments induced the synthesis of 154 kD and 275 kD polypeptides when alfalfa roots were exposed to the interaction of 7 mM $CaSO_4$ and 88 mM NaCl and the interaction of 14 mM $CaSO_4$ and 132 mM NaCl, respectively (Fig. 4).

3. There was a termination of 29-kD polypeptide when Centurion was exposed to 7 mM $CaSO_4$ and 132 mM NaCl for 6 days (Fig. 5).

4. Inducement of 36-kD polypeptide was noticed when Condor roots were exposed to a mixture of 7 mM $CaSO_4$ and 132 mM NaCl and 14 mM $CaSO_4$ for 3 days (Fig. 6).

5. Termination of 20.1-kD polypeptide was observed when Condor was exposed to salts without $CaSO_4$ (Fig. 6).

6. Inducement of 17-kD and 14-kD polypeptides was noted when Condor was exposed to 132 mM NaCl for 9 days (Fig. 7).

7. All isolated plasma membranes showed sensitivity to Na_2VO_3 and insensitivity to NaN_3, a marker for mitochondria contaminants (Tables 1, 2, 3, and 4).

8. ATPase activity increased 4.85%, then decreased 5.1% when Centurion roots were exposed to 88 mM and 132 mM NaCl, respectively (Table 1).

9. The addition of $CaSO_4$ increased ATPase activity by 7.5%, and 38.1%, 52.9%, and 43.8% when Centurion was exposed to 7 mM $CaSO_4$ and 88 mM NaCl; 7 mM $CaSO_4$ and 132 mM NaCl; 14 mM $CaSO_4$ and 88 mM NaCl; and 14 mM $CaSO_4$ and 132 mM NaCl, respectively (Table 1).

10. ATPase activity was decreased by 48.9% when exposed to 132 mM NaCl (Table 2).

11. The addition of $CaSO_4$ increased ATPase activity to its normal value obtained by the control (Table 2).

12. ATPase activity increased by 49.5% when Centurion was exposed to 7 mM $CaSO_4$ and 132 mM NaCl; 14 mM $CaSO_4$ and 88 mM NaCl; and 14 mM $CaSO_4$ and 132 mM NaCl (Table 2).

13. ATPase activity decreased by 36.3% and 49.5% when Condor was exposed to 88 mM and 132 mM NaCl, respectively (Table 3).

14. ATPase activity increased by 26.7% when Condor was exposed to 14 mM $CaSO_4$ and 88 mM NaCl, and to 14 mM $CaSO_4$ and 132 mM NaCl (Table 3).

15. ATPase activity decreased then increased when Condor was exposed to 88 mM and 132 mM NaCl, respectively (Table 4).

16. ATPase activity increased 59.7%, 70.8%, 54.2%, and 59.7% when Condor was exposed to 7 mM $CaSO_4$ and 132 mM NaCl; 7 mM $CaSO_4$ and 132 mM NaCl; 14 mM $CaSO_4$ and 88 mM NaCl; and 14 mM $CaSO_4$ and 132 mM NaCl, respectively (Table 4).

Determination of Na, Ca, Mg, and K levels was made according to Cramer and Spurr's method (22). The results obtained are as follows:

1. Na levels were lower in 9 days than in both 3 days and 60 days (Fig. 8).

2. Na levels were higher when Centurion was exposed to 88 mM and 132 mM NaCl than when exposed to 7 mM and 14 mM $CaSO_4$ (Fig. 8).

3. Na levels in Condor were more than 4 meq/L, while Na levels in Centurion exceeded 11 meq/L (Fig. 9).

4. High levels of NaCl decreased the concentrations of Ca (Fig. 11)

5. Addition of $CaSO_4$ improved K levels in Centurion and Condor (Figs. 12 and 13).

6. Addition of $CaSO_4$ improved Mg levels in Centurion and Condor (Figs. 14 and 15).

Conclusions

Many proteins may have been either discarded throughout the isolation steps of plasma membrane or degraded by proteases (34). This may account for the appearance of small molecular weights not being observed during electrophoresis despite the fact that relatively high levels of ions were obtained from the roots. Alternatively, it may lead us to conclude that salinity caused some plasma membrane perturbation by ionic strength, which affects Ca displacement or specific effects on membrane receptors (1). Supplemental calcium, however, ameliorated the effects of salinity by increasing Ca entry and, thus, improving K and Mg status while reducing Na entry.

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APPENDICES

Appendix A:

Modified Hoagland Solution

Table 5. Chemical contents of the modified Haogland nutrient solution.

Chemical	Conc.	ml L^{-1}
$Ca(NO_3)_2$	1 M	5
KNO_3	1 M	5
$MgSO_4$	1 M	2
KH_2PO_4	1 M	
H_3BO_3	500 ppm	1
$MnCl_2.4H_2O$	500 ppm	1
$ZnSO_4.7H_2O$	500 ppm	1
$CuSO_4.5H_2O$	20 ppm	1
MoO_3	10 ppm	1
Fe Chelate	4 g chelate L^{-1}	10

Appendex B:

Bradford Protein Assay

Bradford protein assay:

1. Set-up 6 tubes in a rack -- get 1 mg mL^{-1} BSA from refrigerator.

2. Using a Hamilton syringe -- put 20 uL, 40 uL, 60 uL BSA into each of 3 tubes -- add ddH_2O and 2 sample tubes:

100 uL H_2O	80 uL H_2O 20 uL BSA	60 uL H_2O 40 uL BSA	40 uL H_2O 60 uL BSA	80 uL H_2O 20 uL prep
1	2	3	4	5 6
Blank	Standards			Sample tubes

3. Filter Bradford reagent through Whatman #1 paper and add 5 mL to each tube -- vortex and let sit 10 min.

4. Read absorbance on spectrophotometer at 595 nm. Need to do regression analysis on standards.

Appendex C:

Ohnishi Assay for Inorganic Phosphate

Steps followed for Ohnishi assay:

1. Prepare assay tubes (on ice) with the following:

Solution	Vol/assay (mL)
0.3 M Tris/Mes (pH 6.5)	0.1
15 mM $MgSO_4$	0.2
0.5 M KCl (or KCl)	0.1 (0.0)
15 mM ATP	0.2
H_2O	0.3 (0.4)

2. Additions -- i.e., If assaying solubilized -- reconstituted preparation, add 3 μL of 2 mM Gramicidin and reduce water accordingly -- final tube concentration is 1 mL.

3. Timed: Add 0.1 mL (100 μL) of prep solution (ATPase) -- Protein concentration = 25 μg/mL. Vortex and place in 37° C water bath for 20 min. Run 1 tube ever 10-15 s.

4. Timed: Stop reaction by adding 5 mL of mixed reagent in 3:2:1 ratio of B:A:H_2O.

5. Timed: After 2 min, add o.5 mL reagent C (color developer). Let stand for at least 10 min. Read tubes at 720 nm.

6. Run 1 blank (no ATPase) and standards: 0.1, 0.3, etc.

7. Reagents:

	250 mL	500 mL
a. Molybdate Preservative. (Make fresh every week)		
$(NH_3)Mo_7O_{24}4H_2O$	10.00 g	20.00 g
EDTA-Na_4 (free acid)	1.52 g	2.32 g
b. Reductant.		
Hydroxylamine sulfate	7.058 g	14.12 g
PVP-40	10.00 g	20.00 g
H_2SO_4	1.23 mL	2.46 mL
c. Color Developer		
Na Carbanate	1.32 g	2.64 g
NaOH	64.7 g	129.4 g

Appendex D:

Modified Silver Stain

Stock Reagent:

50% (Commercial Glutaraldehyde-should be reagent but not necessarily EM grade.

Reagent grade ethanol (100%) and methanol

0.36% sodium hydroxide

concentrated acetic acid

concentrated NH_3OH - should be fairly fresh

TCA

19.4% (w/v) $AgNO_3$ (store in brown bottle)

1% (w/v) citric acid

37% (commercial) formaldehyde

Procedure: (for one gel 0.05-0.075 x 14 x 14 cm)

Step	Solution	Vol.	Time
1. Fixation #1	10% TCA, 5% acetic acid, 30% methanol	50 ml	30 min
2. Fixation #2	2.5% Glutaraldehyde	50 ml	30 min
3. Rinse (3 times)	dH_2O	200 ml	10 min
4. Overnight rinse	10% ethanol (can use 95% ethanol instead of 100% ethanol	400 ml	3 hr
5. Diammine solution step	titrate 2 ml of of 19.4 $AgNO_3$ with base solution (10 ml 0.36% NaOH + 0.67 ml conc NH_3OH-fresh) until red precipitate diappears, and add 1.5 ml additional base. Then combine with ethanol solution (5 ml ethanol + 37 ml dH_2O)	50 ml	12-15 min
6. Rinse	dH_2O	200 ml	5 min

7. Reducer step	0.005% citric acid, 0.0185% formaldehyde, 10% ethanol (i.e. 0.5 ml 1% citric acid, 0.05 ml commercial formaldehyde, and 10 ml ethanol in 100 ml	75 ml	until stain to desired
8. To stop reaction add 100 ml of 1% citric acid			
9. Rinse (3 times)	dH_2O	100 ml	about 10 min

Appendix E:

Analysis of Variance Tables

Table 6. Analysis of variance for Na concentration in roots of alfalfa plant.

Source	df	MS	F
Treat. (T)	6	101.007	261.676**
Control vs rest	1	109.434	283.508**
NaCl (N)	1	10.596	27.451**
$CaSO_4$ (C)	2	238.736	618.487**
N x C	2	4.269	11.060**
Error (a)	14	0.386	
Cult. (A)	1	222.780	527.915**
T x A	6	18.457	43.737**
N x A	1	27.068	64.142**
C x A	2	31.954	75.720**
N x C x A	2	0.775	1.836
Error (b)	14	0.422	
Exposure (E)	2	12.734	14.324**
Error (c)	4	0.889	
T x E	12	5.323	13.614**
N x E	2	0.688	1.760
C x E	4	8.085	20.678**
N x C x E	4	1.322	3.381*
A x E	2	1.668	4.266*
T x A x E	12	2.510	6.421**
N x A x E	2	1.071	2.739
C x A x E	4	0.669	1.711
N x C x A x E	4	3.649	9.332**
Error (d)	52	0.391	
Total	125		

*, ** Significant at 0.05 and 0.01 propability levels, respectively.

Table 7. Analysis of variance for Ca concentration in roots of alfalfa plant.

Source	df	MS	F
Treat. (T)	6	104.8453	3826.471**
Control vs rest	1	58.217	2124.697**
NaCl (N)	1	15.240	556.204**
$CaSO^4$	2	275.318	10048.102**
N x C	2	2.489	90.839**
Error (a)	14	0.0274	
Cult. (A)	1	0.0201	0.791*
T x A	6	0.0254	1.000
N x A	1	0.000	0.000
C x A	2	0.030	1.181
N x C x A	2	0.038	1.496
Error (b)	14	0.0254	
Exposure (E)	2	1.9602	292.567**
Error (c)	4	0.0067	
T x E	12	1.8217	239.697**
N x E	2	1.077	141.711**
C x E	4	3.396	446.842**
N x C x E	4	0.772	101.579**
A x E	2	1.098	144.474**
T x A x E	12	0.624	82.105**
N x A x E	2	0.760	100.00**
C x A X E	4	0.706	92.895**
N x C A X E	4	0.742	97.632**
Error (d)	52	0.0076	
Total	125		

*, ** Significant at 0.05 and 0.01 propability levels, respectively.

Table 8. Analysis of variance for K concentration in roots of alfalfa plant.

Source	df	MS	F
Treatment (T)	6	18.898	245.429**
Control vs rest	1	108.32	1406.753**
NaCl (N)	1	1.193	15.494*
$CaSO_4$ (C)	2	0.896	11.664*
N x C	2	1.041	13.519**
Error (a)	14	0.077	
Cult (A)	1	282.080	2765.49**
T x A	6	15.7014	153.936**
N x A	1	0.135	1.323
C x A	2	0.000	0.000
N x C x A	2	1.031	10.108**
Error (b)	14	0.102	
Exposure (E)	2	26.219	137.344**
Error (c)	4	0.191	
T x E	12	5.253	47.324**
N x E	2	21.048	189.622**
C x E	4	0.218	1.964
N x C x E	4	1.481	13.342**
A x C	2	27.591	248.568**
T x A x E	12	5.245	47.252**
C x A x E	4	0.105	0.956
N x A x E	2	20.416	183.928**
N x C x A x E	4	1.668	15.027**
Error (d)	52	0.111	
Total	125		

*,** Significant at 0.05 and 0.01 propability levels, repectively.

Table 9. Analysis of variance for Mg concentration in roots of alfalfa plant.

Source	df	MS	F
Treat. (T)	6	3.898	397.755**
Control vs rest	1	0.439	44.796**
NaCl (N)	1	5.333	544.184**
$CaSO_4$ (C)	2	7.558	771.224**
N x C	2	1.248	127.347**
Error (a)	14	0.0098	
Cult. (A)	1	5.824	1294.222**
T x A	6	0.758	168.444**
N x A	1	1.396	310.222**
C x A	2	0.734	163.111**
N x C x A	2	0.599	133.111**
Error (b)	14	0.0045	
Exposure (E)	2	13.191	57352.174**
Error (C)	4	0.00023	
T x E	12	1.952	323.715**
N x E	2	1.171	194.196**
C x E	4	3.895	645.937**
N x C x E	4	0.222	36.816**
A x E	2	2.449	406.135**
T x A x E	12	0.399	66.169**
N x A x E	2	0.387	64.179**
N x A x E	4	0.764	126.700**
N x C x A x E	11	0.182	30.333**
Error (d)	52	0.00603	
Total	125		

** Significant at 0.01 propability level.

Table 10. Analysis of variance for K^+/Na^+ ratio in roots of alfalfa plant.

Source	df	MS	F
Treat. (T)	6	6.7131	293.148**
Control vs rest	1	34.6291	1512.188**
NaCl (N)	1	0.0435	1.900
$CaSO_4$ (C)	2	2.7142	118.524**
N X C	2	0.0889	3.882*
Error (a)	14	0.0229	
Cult. (A)	1	3.3281	170.672**
T X A	6	3.2268	165.477**
N X C	1	0.1275	6.538*
C X A	2	3.2516	166.749**
N X C X A	2	0.4601	23.595**
Error (b)	14	0.0195	
Exposure (E)	1	3.4729	482.347**
Error (c)	2	0.0072	
T X E	6	0.8046	27.649**
N X E	1	0.1005	3.454
C X E	2	0.6669	22.918**
N X C X E	2	0.9984	34.309**
A X E	1	5.4621	187.701**
T X A X E	6	0.6262	21.519**
N X A X E	1	0.0678	2.330
C X A X E	2	1.3814	47.471**
N X C X A X C	2	0.2260	7.766*
Error (d)	26	0.0291	
Total	83		

*,** significant at 0.05 and 0.01 propability levels, respectively.

Table 11. Analysis of variance for Ca^{2+}/Na^{+} ratio in roots of alfalfa plant.

Source	df	MS	F
Treatment (T)	6	44.401	240.005**
Control vs rest	1	0.360	1.946
NaCl (N)	1	25.205	136.243**
$CaSO_4$ (C)	2	109.795	583.486**
N X C	2	10.625	57.432**
Error (a)	14	0.185	
Cult. (A)	1	6.983	44.196**
T X A	6	9.176	58.076**
N X A	1	6.266	39.658**
C X A	2	19.100	120.886**
N X C X A	2	3.100	19.620**
Error (b)	14	0.158	
Exposure (E)	1	90.273	1074.679**
Error (c)	2	0.084	
T X E	6	50.262	366.876**
N X E	1	75.522	551.255**
C X E	2	47.244	344.847**
N X C X E	2	23.705	173.029**
A X E	1	19.296	140.847**
T X A X E	6	6.515	47.555**
N X A X E	1	15.033	109.730**
C X A X E	2	5.961	43.511**
N X C X A X E	2	4.240	30.949**
Error (d)	26	0.137	
Total	83		

** significant at 0.01 propability level.